Approvals		
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Identification and Enumeration of Marine and Estuarine Benthic Invertebrates

1.0 Scope and Application

1.1 The identification and enumeration of marine and estuarine benthic invertebrates is done in support of biological assessments in coastal waters of EPA Region III in which a description of the benthic community is to be used as an indicator of environmental quality. This analysis includes the primary identification and enumeration of invertebrates for assessment projects and requested quality control identification and enumeration of specimens already identified and enumerated by external (e.g., state) taxonomists. Depending on the requirements of the project, taxa may be identified down to anyone of several taxonomic levels (e.g., family, genus, species, or lowest possible taxon). Samples are received either already sorted (vials received contain specimens only) or needing picking and gross sorting. The latter has been performed by this laboratory (see SOP R3-QA500.0)

2.0 Summary of Procedure

2.1 Specimens to be identified are examined under either a stereo-dissecting scope or a compound microscope, or both, depending on the characters that need to be observed. Direct counts of specimens are made, or in the case of large abundances of one taxon subsampling is performed and total counts of that taxon estimated

2.2 Quality control measures for each project include a reference (or voucher) collection and a ten percent (ten percent of samples) check of identifications and counts by a second taxonomist.

3.0 Definitions

3.1 Benthic Invertebrates. The animals to be processed through identification and enumeration will ultimately be determined by a project plan. Typically the benthic invertebrates include all those multicellular animals not belonging to the chordate subphylum Vertebrata, dwelling on or in the bottom, and retained on a 500 micrometer mesh sieve (or 1.0 millimeter mesh depending on project plan). Excluded are minute

taxa that have been traditionally by convention identified as belonging to the meiofauna. These would include nematodes, ostracods, kinorhynchs, tardigrads and others.

3.2 Lowest possible taxon. The lowest level possible given the condition and age or maturity of specimens. If specimens are in good condition with all necessary adult taxonomic characters intact, lowest possible taxon would mean species level.

3.3 Sample. This term has been defined and used (or misused) in various ways in the benthic ecology literature. However, for the purposes of this document and unless defined otherwise by a particular project plan, a sample is defined as a single benthic grab (e.g., Ponar grab) and is considered synonymous with the term "replicate".

4.0 Interferences

[Not applicable.]

5.0 Safety

5.1 The analyst must know and practice the ESC facility safety rules and procedures.

5.2 All applicable safety and compliance guidelines set forth by the EPA and by federal, state, and local regulations must be followed during the performance of this SOP. In addition, all procedures outlined in the OASQA Chemical Hygiene Plan (CHP) must be adhered to. Stop all work in the event of a known or potential compromise to the health and safety of any person and immediately notify the Chemical Hygiene Officer and other appropriate personnel as outlined in the CHP.

5.3 All laboratory waste must be handled in accordance with guidelines established in the CHP and the appropriate waste disposal procedures identified in Section 15 (Waste Management).

5.4 All specimens preserved in 70% EtOH are to be stored in a flammable materials safety cabinet .

5.4 The specimen preservative, 70% EtOH, is to be used and stored under a hood.

5.5 Although all specimens are typically preserved in 70% EtOH, occasionally large-bodied specimens (e.g., the estuarine clam, *Rangia cuneata*) may leach trace amounts of the formaldehyde used as a fixative. When this occurs all work with these specimens must be performed under a hood and hands must be protected with gloves. The resulting waste (70% EtOH, wash water, trace amounts of formaldehyde and Rose Bengal stain) must be stored in a labeled waste container within the hood. See waster stream diagram (attachment). [waster stream not yet drafted].

6.0 Equipment and Supplies

6.1 stereo-dissecting microscope (Olympus SZ60, Zoom Objective: 1 - 6.3x)
6.2 compound microscope with phase contrast optics (Olympus BX50, Objectives: 4x, 10x, 20x, 40x, 100x)
6.3 fiber optic illuminators (dual gooseneck illuminators)
6.4 95% EtOH (diluted as necessary to produce approximately 70% EtOH)
6.5 glycerin (for preparation of glycerin:EtOH (1:1) temporary mounting medium)
6.6 dropper bottle (for dispensing glycerin:EtOH mounting medium).
6.7 forceps (very fine-tipped)
6.8 observation or petri dishes (various sizes)
6.9 vials and jars (various sizes)
6.10 plastic disposable pipettes
6.11 glass coverslips (22 x 22 mm)
6.12 Nalgene squirt bottles
6.13 small sieve (stainless steel, 8 cm dia., 250 micrometer mesh)
6.14 mechanical counter
6.15 waterproof paper (for labels)
6.16 datasheets
6.17 library of literature (books and journal article reprints) on marine/estuarine benthic invertebrates. (aboratory's library contains over 900 reprints.)
6.18 PC (capable of running bibliographic search software)
6.19 *Polychaete Literature Bibliographic Database* (searchable database containing 17,000 entrys loaded on C: drive), L. Ward and K. Fauchald (1996) National Museum of Natural History, Smithsonian Institution.
6.20 *Zoological Record*, 1978-1999 (bibliographic database on CDs)

7.0 Reagents

7.1 The approximately 70% EtOH used as a preservative is produced by diluting 95% EtOH with tap water.

7.2 The 1:1 glycerin:EtOH temporary mounting medium is produced by adding equal amounts of undiluted glycerin and 95% EtOH.

8.0 Sample Collection, Preservation, and Storage

8.1 Sample collection should be done according to the QAP prepared by the principal investigator for the project (external to OASQA). Guidance for sample collection can be found in the references listed below in Section 16.0.

8.2 Although this process is not done in this laboratory, benthic invertebrates samples should be fixed for at least 24 hours with 10% formaldehyde, washed well with tap water, and preserved with 70% EtOH.

8.3 Specimens for taxonomic analysis preserved and held in 70% EtOH must be stored in a flammable materials storage cabinet.

9.0 Quality Control

9.1 These QC measures apply to the primary taxonomic analysis performed in support of bioassessments, and not to the QC performed on the work of an external taxonomic laboratory.

9.2 A reference (or voucher) specimen collection must be prepared for each project, and in the case of long term multi-year projects, for each year of sampling. A reference specimen should be in good condition, exhibiting critical adult characters. Each reference specimen must be placed in its own vial (70% EtOH) with a label (waterproof paper) containing date collected, general geographic area (e.g.,"Chester River"), sample station and sample (or replicate) identification, and initials of taxonomist who identified specimen and designated it as a reference specimen. This information must be recorded in pencil on the label. If specimens exhibit variation in a character or characters critical to its identification, several specimens of the taxon should be placed in the vial. A log will be kept of all specimens in the reference collection; see 10.6 below.

9.3 Upon completion of a batch of samples (10 samples), one sample (10% of the samples) is checked to verify accuracy of species identifications and counts by a second taxonomist. This control check confirms the level of accuracy with which identification and counts are performed and offers feedback to taxonomists in the laboratory needed to maintain a high standard of accuracy. Samples will never be re-checked by the taxonomist who originally processed the sample. The second taxonomist should have experience at least equivalent to (and preferably greater than) that of the taxonomist who performed the original identifications and counts. Ideally, a sample batch is made up of samples from a similar habitat type (e.g., all samples from the oligohaline habitat). The one sample to be checked must be selected at random from the sample batch. All specimens regardless of taxonomic category must be re-identified and re-counted without knowledge of the original data.

9.4 As each specimen included in the 10% check is re-identified and re-counted, the results are recorded in a QC logbook and compared to the original data. Discrepancies will be double-checked to verify that the final QC result is correct. The QC logbook contains 1) the samples included in the sample batch, 2) the sample that is checked, 3) the results of the QC check, 4) the original data, 5) misidentifications (clearly indicated), 6) a calculation of percent error (see 9.5), 7) the date and initials of the taxonomist performing the QC check, and 8) any corrective actions that were taken.

9.5 When the entire sample has been re-identified, the total number of errors will be determined and a percent error calculated. Percent error will be calculated in the following manner:

$$\text{Percent Error} = \frac{\text{Total \# of Errors}}{\text{Total \# of Individuals in QC Recount}} \times 100$$

Two types of errors are included in the total number of errors:

1) Counting errors (for example, counting 12 *Rangia cuneata* as 10 represents 2 errors)

2) Identification errors (for example, identifying a *Leptocheirus plumulosus* specimen as *Melita nitida*, represents 1 error, even though count of *M. nitida* will change.)

9.6 The maximum acceptable percent error will be 10%. Regardless of the percent error calculated, all errors will be communicated to the original taxonomist. Percent errors > 10% will require re-identifying and re-enumerating all samples in the sample batch. Taxonomists must be particularly sensitive to systematic errors (i.e., repeated errors for specific taxonomic groups) that may suggest the need for further training

9.7 Changes in identifications or counts resulting from QC procedures, will be recorded on the original datasheet with a single line through old result, the new result written above or to the side, and the date and initials of person making the change.

10.0 Standardization

10.1 For the purpose of standardization and consistency over time and among taxonomists across the region, each project reference specimen collection will be reviewed by an external taxonomist working in the region. Results of that review and a record of corrective actions taken will be kept on file.

10.2 Informal interaction on taxonomic issues among taxonomists working in the region should be maintained.

10.3 Reference specimens belonging to particularly difficult or controversial taxonomic groups, should be sent to recognized experts for verification.

10.4 The reference collections should be used to train new taxonomists to ensure standardization within the laboratory.

10.5 Reference specimens should be checked periodically for loss of ethanol and topped-up if necessary.

10.6 For each reference collection generated, a log will be maintained containing the taxon's name, number of specimens, station and replicate, initials of taxonomist who identified voucher specimens, date entry recorded, references used (author and date) to make identification, and any pertinent miscellaneous notes, including information, if any, about the confirmation of the identification by outside experts. A copy of the log will be placed in the final case file.

11.0 Procedure

11.1 The objective of species identification and enumeration is to accurately identify all animals found in a sample to the lowest possible taxonomic category consistent with the project objectives and to accurately count the number of animals in each such category. Project objectives and expectations as to the lowest possible taxonomic categories to

pursue for different groups of invertebrates must be decided upon ahead of time by the principal investigators. These should be indicated in a project plan.

11.2 All identifications are performed under a high quality stereo-dissecting microscope, a high quality compound microscope, or both, as is often necessary.

11.3 A specimen, or portion there of, is examined with the compound microscope first by placing a drop or two of glycerin:EtOH (1:1) on a microscope slide, placing the specimen in the glycerin:EtOH and carefully adding a glass coverslip at an angle to minimize the bubbles trapped under it.

11.4 The number of individuals counted for each taxon must reflect the number of organisms alive at the time of sampling. When fragments are found, only head (or anterior end) fragments will be counted. Empty bivalve or gastropod shells will not be counted. Intact closed bivalve shells (e.g., *Rangia cuneata*) must be cracked or opened to confirm that a recently living animal resides inside.

11.5 As the processing of a new sample for identification and enumeration begins, a species identification datasheet is started. The station and sample (or replicate) number are recorded on the datasheet. All specimen identifications for all replicates from one station are recorded on a single datasheet. Upon completion of a station's datasheet, the datasheet must be initialed and dated by the taxonomists who performed the identifications.

11.6 Identification and enumeration proceeds by higher taxonomic category organized by station and sample (replicate). For example, all crustaceans will be examined first sample by sample, followed by all annelids, sample by sample. As each new vial is analyzed the taxonomist will double-check that a datasheet has been initiated for that station, and if not, will start one.

11.7 If samples are from an area of known high benthic invertebrate diversity or an unfamiliar area, the basic approach is to identify all specimens to progressively lower levels, one level at a time. For instance, all polychaetes might be first identified and grouped in families for all samples, then subsequently identified to genus and species.

11.8 If samples are from a familiar estuarine area characterized by relatively low benthic species diversity, specimens may be identified to the lowest possible taxon immediately.

11.9 At all times, identifications are to be made with consideration given to the most recently published scientific literature. The two electronic bibliographic databases in the laboratory (*Polychaete Literature Bibliographic Database* and *Zoological Record* – see 6.0 above) will be used for this purpose.

11.10 Specimens from each vial will be transferred using forceps or pipettes from the vial (70% EtOH) to tap water in an observation dish (small petri dish). Alternatively, in the case of numerous specimens in a single vial, the specimens are to be washed with tap water into a small sieve (mesh opening 250 micrometers) and then washed from the sieve with tap water into a petri dish.

11.11 Specimens will be identified, counted, and transferred from the petri dish to 70% EtOH. Depending on project requirements identified specimens will be grouped back into the higher category vials or sorted into vials for each of the lowest taxonomic categories (e.g., species) to which they were identified.

11.12 Specimens to be placed in the project's reference specimen collection are to be transferred into a separate vial with the appropriate label (see 9.2 above). These specimens are to be included in the count recorded on the datasheet; however, the count is to be tagged with an asterisk referring to a footnote at the bottom of the datasheet indicating the number of specimens of that taxon diverted into the project reference collection.

11.13 Petri dishes and sieves will be thoroughly inspected for missed specimens and rinsed well to minimize cross contamination between vials and samples. Sieves will be rinsed with tap water from the underside of the sieve.

11.14 Identified specimens will be checked for suspicious mismatches between taxon and habitat (e.g., the identification of a polyhaline polychaete species in a sample from an oligohaline station).

11.15 If the number of specimens of a taxon exceeds 100, the sample may be subsampled in the following manner. Specimens will be distributed randomly in a glass tray placed on top of a black on white grid. Grid squares will be selected at random until half the total number of grid squares are selected. Specimens will be removed (with forceps or a disposable plastic pipette if necessary) from the selected grids, identified, and counted. This count will be multiplied by two to achieve the estimated count for the sample. When the estimated count is recorded an asterisk will refer to a footnote, explaining that the count is an estimate based on subsampling according to this SOP and specifying the number of grid squares (subdivisions) counted and the total number of squares comprising the grid used.

11.16 In the case of recently revised taxonomic groups or controversial taxonomic designations (i.e., where authors disagree on the correct classification and nomenclature for a particular group) the taxonomist will use his or her best professional judgement in assigning a taxon name. And, due to the existing controversy (or lack of clarity in the literature), the taxonomist will add the author's last name and date of publication to the name recorded on the datasheet and on the reference specimen label. Likewise, if the taxonomist encounters a species believed to be new to science (i.e., not previously described in the literature), the taxonomist will use the designation "sp. A" (B, C, etc. as necessary) followed by the last name of the taxonomist and date.

11.17 If identifications are to be done to species level, specimens that cannot be identified to that level due to poor condition or immaturity, will be recorded as identified to the family level (or other appropriate higher category) with the designation of "species indeterminable" (abbreviated "sp. indeter." or "sp. indet."). Typically this occurs for damaged or incomplete anterior fragments or juvenile specimens. For example, poor or juvenile specimens of the polychaete family Spionidae, would be counted and recorded as "Spionidae sp. indeter.".

12.0 Data Analysis and Calculations

[Not applicable since raw data is product delivered to customer. Calculations for QC percent error and subsampling are noted above.]

13.0 Method Performance

[See QC calculation of percent error, paragraph 9.5.]

14.0 Pollution Prevention

14.1 As this method is routinely performed, the analyst is to consider steps to continually reduce the use and generation of hazardous chemicals. This consideration should include minimization of the amount of chemicals purchased and recycling the 70% EtOH when possible.

15.0 Waste Management

15.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

15.2 Points of waste generation and disposal for the procedures described above are indicated in the attached waste stream diagram. [waste stream yet to be drafted].

16.0 References

16.1 Clesceri, L. S., A. E. Greenberg and A. D. Eaton (eds.), 1998. *Standard Methods for the Examination of Water and Wastewater,* 20th Edition. APHA, AWWA, WPCF. Sections 10500 and 10900 .

16.2 Strobel, C.J., D.J. Kemm, L.B. Lobring, J.W. Eichelberger, A. Alford-Stevens, B.B. Potter, R.F. Thomas, J.M. Lazorchak, G.B. Collins, and R.L. Graves. 1995. *Environmental Monitoring and Asssessment Program (EMAP)- Estuaries: Laboratory Methods Manual, Vol.1 - Biological and Physical Analyses.* Office of Research and Development, U.S. Environmental Protection Agency, Narragansett, RI.